Imaging Mouse Embryonic Development

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1 Goal

Vertebrate development is a dynamic process that requires the careful orchestration of many events. In a relatively short amount of time cells are added, deleted, transformed and assigned roles that can be vital to the survival of the organism. Through sophisticated genetic manipulation and examination of naturally occurring mutations, hundreds of genes have been identified that play key roles in early mouse development. Understanding these roles fundamentally relies on understanding the phenotypic consequences of the mutations.

In many organisms that develop external to the mother, vital imaging has been used to determine the relationships between cells that are involved in rapid morphogenetic processes (for review, see Lichtman and Fraser 2001). Since development is a continuous process, much more information about cellular phenotypes can be gained through continuous observation. Because mouse embryos develop in utero, most of what is known about complex phenotypes stems from embryos collected and fixed at several time points. Though culture and imaging of pre-implantation embryos has been possible for some time now, post-implantation embryos have traditionally been cultured in roller culture, which does not permit time-lapse analysis of embryonic development using a microscope. To circumvent these limitations, we have developed a technique for static culture on a microscope stage that allows dynamic imaging of embryos from gastrulation until early organogenesis (Jones et al. 2002, Hadkantonakis, A.K. & Papaioannou, V.E., unpubl.). Whole embryo culture, combined with stable fluorescent markers, such as Fluorescent Protein variants, that can easily be introduced via existing methods, provides a powerful tool for understanding the cellular consequences of genetic manipulation in mice.

2 Area of application- Imaging Mouse Development

The development of the mouse embryo has been described in many volumes (including (Theiler 1989; Rugh 1990; Kaufman 1992) and there are currently strong efforts to digitize atlases of fixed

stages of development, making them more accessible and interactive. These atlases and descriptions of mouse development have been invaluable to understanding normal ontogeny as well as mutant phenotypes, but information about dynamic processes such as cell lineage or cell migration cannot be fully revealed from static images. For these reasons, methods for manipulating embryos ex vivo have been developed.

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Protocols for culturing pre-implantation embryos have been available for some time and has not only provided great insight into early cleavage events, but has also been the cornerstone of transgenic and gene knock-out technologies (Nagy 2003). Imaging early post-implantation development is more difficult. Embryos at these stages require a rich supply of freshly prepared serum and have traditionally been grown in roller culture (Tam 1998). The constant movement of the media in roller cultures promotes gas exchange and since the flow rate of gas is minimal, evaporation is limited. The protocol below shows how similar conditions can be used to culture mouse embryos on the stage of the microscope for fluorescence imaging. This protocol is recommended for mouse embryos from 6.5 to 9.5 dpc for 18-24 hours. After 10.0 dpc, embryos become far more difficult to maintain using this method, although others have described a serum-free protocol for growing mid-gestration stage embryos in culture (Moore-Scott et al. 2003). These stages have also been studied successfully by labeling cells in embryos that are allowed to develop exo utero (Muneoka et al. 1986; Turner et al. 1990; Serbedzija et al. 1992; Saito and Nakatsuji 2001).

Many of the labeling techniques developed for other developmental models are applicable to mouse embryos grown in culture, including dye injection, tissue grafting (Quinlan et al. 1999) and retroviral labeling. The strength of the mouse model, however, is the ability to manipulate the genome in such a way that genetically encoded fluorescent proteins can be expressed in specific cells or tissues (Figure 1). In fact, several genetic strains have been engineered to express fluorescent proteins in defined regions of the embryo. For example, the Oct4 promoter has been

used to drive fluorescence in the primordial germ cells (Anderson et al. 1999). The ε-globin promoter has been used to express GFP only in the primitive erythroblasts (Dyer et al. 2001) and the Hex promoter can be used to specifically label the AVE (Rodriguez et al. 2001). In addition, many knock-ins of fluorescent proteins exist that combine fluorescence imaging with mutant analysis, such as the Nkx2.5$^{GFP/GFP}$ (Biben et al. 2000) or the Tbx6 mutant, which labels the presomitic mesoderm and newly formed somites (Hadjantonakis and Papaioannou, unpubl.). As well, the use of Internal Ribosome Entry Sites (IRES) can be used to achieve single copy protein expression (Rodriguez et al. 1999).

Though most current transgenics express fluorescent proteins throughout the cytoplasm of the cells of interest, fluorescent proteins that are specifically associated with distinct subcellular regions have also been produced (Figure 2). In some cases, localization of the protein is conferred by engineering fusion proteins or by tagging with sequences recognized by protein trafficking machinery. Multiple-color transgenics can be made to understand whether signal and putative responsive cells interact. With the advent of convenient tools for multispectral analysis and linear unmixing, such as those available on the Zeiss LSM 510 META, it is now possible to image labels with multiple colors without spectral bleed-through, even if the emission spectra are highly overlapping (for more info, see (Dickinson et al. 2001; Lansford et al. 2001; Dickinson et al. 2003). By multiplexing, more complex interactions between cells in the developing embryo can be understood.

3 Materials

3.1 Media

Two types of media are used in mammalian embryo culture; a dissection medium and a culture medium.

The dissection medium is composed of 90 vol% D-MEM/F-12 (Gibco), 8 vol% heat-inactivated fetal bovine serum (Gibco), 1 vol% HEPES buffer solution 1M (Irvine Scientific) and 1 vol% Pen-Strep solution (Irvine Scientific). This medium must be heated to 37°C before use in a water bath.

The culture medium depends on the stage of the embryo. For younger embryos (E5.5 to E7.5), the media consists of 50% D- MEM and 50% rat serum. For embryos between E7.5 and E9.5, D-MEM/F12 is used instead of D-MEM and the culture medium is supplemented with 10 μL/mL HEPES buffer and 10 μL/mL Penicillin-Streptomycin. The media is filtered through a 0.2 μm filter to sterilize it. At all stages, it is necessary to heat and equilibrate the pH of the media by placing it in a tissue culture incubator (5% CO_2, 37°C) for at least an hour.

The most important component to proper mammalian embryo culture is the use of high quality rat serum. Though some commercial sources exist, we find that home-made preparations give consistently superior results. The following protocol is used for the collection of rat serum:

1. Anesthetize male rats with ether.
2. Spray the abdomen with 70% ethanol and make a v-shaped incision into the lower abdomen.
3. Expose the dorsal aorta, which is located next to the larger vena cava. It is the smaller (around 1 mm diameter) pulsing blood vessel (Figure 3).
4. Puncture the aorta using the butterfly needle. It is simplest to use a butterfly needle connected to a syringe needle (Vaculock system, BD Biosciences). Once the dorsal aorta has been punctured with the butterfly needle, the syringue needle is pressed into a vacutainer tube (BD Biosciences), creating a mild suction to collect the blood.
5. Invert the vacutainer tubes a few times during collection.
6. Once the rat is exsanguinated, the collected blood is placed on ice.

7. Euthanize the rat and allow the ether to evaporate from the carcasses. Each rat gives approximately 2-3 mL of rat serum, therefore process as many rats as needed for your experiments.
8. Centrifuge the blood at 1300g for 20 minutes.
9. Remove and pool the supernatant.
10. Centrifuge the serum at 1300g for 10 minutes to remove any remaining cells and debris and collect the supernatant.
11. Heat-inactivate the serum in a water bath at 56°C for 30 minutes with the lid partially unscrewed to allow the ether to evaporate.
12. Filter the serum using a 0.45 μm filter and aliquot.
13. Freeze aliquots and store at –80°C for up to one year.

It is essential that the blood be collected under low suction. At higher suction, the red blood cells can lyse, reducing the quality of the serum. It is possible to use a syringe for blood collection, rather than vacutainer tubes, however if the plunger is pulled back too quickly, the serum will be unusable due to hemolysis.

The use of ether is also essential to the procedure. Other anesthetics remain in the blood and can affect embryonic growth. Ether, however, will easily evaporate from the serum during heat-inactivation.

3.2 Microscope Setup

Proper environmental control of the microscope stage is probably the single most important factor for successful culture. Mammalian embryos are especially sensitive to small changes in temperature (± 3°C) and small amounts of evaporation. In order to keep the embryos at 37°C, a

heater box must be made that can fit around the microscope stage (Figure 4) or one can be purchase from a commercial manufacturer (e.g. Zeiss).

3.2.1 Construction of a heater box

The heater box is constructed to surround the stage and all the microscope optics. Thermal drift of the optics can occur if they are not contained within the heater box. A cardboard shell is first constructed and then covered with foil-foil insulation thermal insulation (5/16" thick, Reflectix Co.; Markleville, IN). Hot air is blown into the heater box area using a chicken incubator heater (Lyon Electric Company; Chula Vista, CA). The sides of the box should be independent pieces, held together by Velcro or some other form of clasp to allow repeated assembly/disassembly. The incubator heater can be incorporated in place of one of the side of the heater box, or alternatively, the hot air can be introduced via aluminum ducting. Holes should be made in the walls of the box to allow access to the eyepieces and microscope stage (Figure 4). The chicken incubators have built-in temperature controller, however, if more precise temperature control is desired, a digital temperature controller (Fischer Scientific; Springfield, NJ) can be used that will turn the heater on and off.

For imaging on an inverted microscope the embryos are routinely cultured in Lab-tek chambers with coverslip bottoms (Nalge Nunc; Rochester, NY) or 30mm dishes with glass coverslip bottoms (MatTek; Ashland MA). Gas is fed into the environment using 1/8" tubing. A hole is soldered into the lid of the chamber and the gas is connected via a barbed polypropylene fitting (1/16" x 1/8",Cole-Parmer Instrument Co.; Chicago, IL). A more detailed description of this set-up is provided in the procedures section.

The gas requirements for mammalian embryos change depending on the stage of embryonic development. The gas consists of 5% CO_2, variable oxygen concentration, balance nitrogen. For

embryos between E4.5 and E6.5, the oxygen should be kept at 95 %, whereas embryos between E7.5 and E9.5 develop well with 20% oxygen. Since mammalian embryos are sensitive to even the slightest amount of evaporation, several components are required to prevent evaporation. First, the gas is bubbled through a gas-washing bottle (Fisher Scientific; Springfield, NJ). Second, a thin layer of mineral (Sigma; St. Louis, MO) is placed over the media. Lastly, the environment is sealed using silicon grease (Dow Corning; Midland, MI) or Teflon tape. A description of how these components are assembled is provided in the procedures section.

4 Protocol and Procedures

4.1 Dissection

Mice are bred overnight and the presence of a vaginal plug in the morning is taken as 0.5 dpc. On the day of interest, the mouse is euthanized, a v-shaped incision is made into the abominal cavity, and the uterine horns are removed and placed in the warmed dissection media. The muscle is then teased apart and the individual embryos, surrounded by decidua are removed. The deciduum is then carefully removed with Watchmakers No. 5 tweezers (Dumont) to expose the embryo. Standard embryo dissection technique is used as previously been described (Nagy 2003). Reichert's membrane and the trophoblast layer are removed, however all other extraembryonic tissues are left intact up until 9.5 dpc. For very young embryos, the removal of the Reichert's membrane requires the use of a micromanipulator. The ectoplacental cone is usually left on the embryo. The yolk sac can be removed from 8.5 dpc embryos and must be removed from 9.5 dpc embryos.

It is essential that embryos be dissected quickly (less than an hour) and with frequent medium changes. If the medium cools, embryos will not develop normally. If necessary, a heated dissection environment can be used to keep the medium warm during dissection. This can easily be done using heated dissection media and a chicken incubator heater to blow hot air over the

dissection microscope. Since the heated dissection area does not completely prevent the media from cooling, it is important to change the media every 15-20 minutes. If the embryos are exposed to cold media, they take a long time to recover, and often never develop normally.

After dissection, the embryos are transferred to the culture chambers using a pasteur pipette, being careful to transfer as little dissection medium as possible. The culture medium is generally left in the tissue culture incubator to heat during the dissection process. Embryos should not be cultured individually. As well, older embryos (greater than 7.5 dpc) should be limited to three embryos per chamber. Each embryo requires 0.5-1 ml of heated media. Embryos do not develop well if cultured individually or if overcrowded.

The chambers are placed in a tissue culture incubator for at least 1 hr to allow the embryos to recover from dissection. The embryos will develop normally in the tissue incubator heater and can be left for longer if needed.

4.2 Embryo Immobilization

Immobilization of the embryos can be a problem during culture as embryos tend to drift. This is especially significant at higher magnifications. In 8.5 dpc, the yolk sac has expanded and is quite buoyant. This makes the embryos susceptible to small currents in the media and causes to the embryos to orient with the ectoplacental cone, which is heavier, towards the bottom of the dish. At stage 9.5 dpc, the embryos are cultured with the yolk sac removed and therefore sit nicely on the bottom of the Lab-tek chamber.

Embryos can be immobilized by a variety of techniques each serving a different application. At all stages, embryos can be oriented and immobilized using a suction holding pipette attached to a micromanipulator. If culture is modified to use an upright microscope, scratches in the bottom of

the dish can be used to immobilize younger embryos (less than 7.5 dpc). Once the yolk sac has expanded, embryos should be immobilized in any way that does not put pressure on the yolk sac or growing embryo. At 8.5 dpc, even slight pressure from a thin layer of agar or nitex grating overlying the embryo will cause the circulation in the yolk sac to stop. Currently, most embryos are immobilized using a piece of human hair or fine platinum wire, tied in a knot around the ectoplacental cone. The embryos can then be "propped up" in this way. Alternatively, embryos can be immobilized by making a wire hook that is placed around the ectoplacental cone. This can be anchored into wax or agarose, which is pushed into the bottom of the chamber before media is added.

4.3 Time-lapse Imaging

The heater box should be assembled and turned on at least a half hour (though a full hour is preferable) before the time-lapse is to be started in order to let the heater box and microscope optics come to temperature. The gas-washing bottle with a fine diffuser should be placed within the heater box area as well (Figure 4).

The embryos are then placed on the microscope stage and oriented as desired. A thin layer of embryo-tested mineral oil (Sigma) is placed on top of the media, which reduces media evaporation. When using Lab-tek chambers, the bottom of the space between the two chambers is filled with silicon grease. This region will be used for gas inlet to the chamber environment.

The gas outlet from the pressurized cylinder is fed to the washing bottle through appropriate tubing. The regulator on the pressurized cylinder should be set as low as possible since too little gas is not a problem. In order to introduce the gas from the washing bottle into the culture chamber, a small hole is made in the chamber lid. This hole can be made with soldering iron or a hot syringe needle. A small male-male barbed polypropylene fitting is placed within this hole and

tubing is attached to the external end of the fitting to connect the chamber to the gas-washing bottle. Silicon grease is used to ensure the gas inlet is air-tight.

The lid is sealed either with Teflon tape around the outer edges of the lid or with silicon grease. If Teflon tape is used, this must be done before embryos are put on the microscope stage and does not allow re-orientation of the embryos once on the stage. Alternatively, silicon grease is placed around all the inner edges of the chamber lid. With the culture chamber on the microscope stage and the embryos properly oriented, the lid is then placed on top and sealed shut.

For many applications, it is sufficient to image once every 5-10 minutes. Embryos can be sensitive to light exposure and care should be taken to reduce exposure to excitation light. Images can be single frame or, if confocal imaging is being used, several sections can be taken along the z-axis. Culture is much easier at lower magnification, such as 5x, since small shifts in the embryo position do not affect the time-lapse. At higher magnification (up to around 20x), the focus on the embryos will need to be adjusted every hour or two. Using this technique, embryos can be cultured for up to 24 hours for 5.5 to 8.5 dpc, and for 12-18 hours for 9.5 dpc embryos.

During the culture period it is important to prevent any fluctuations in gas flow and temperature. At stages 8.0 dpc and older, embryos should also be observed to ensure the heart rate remains normal. Once culture is complete, the final morphology (as compared to normal in vivo development) of the embryos should be evaluated in order to assess whether development has occurred normally. Most embryos are then fixed in 4% paraformaldehyde in case later analysis is desired.

5 Example of applications

5.1 Anteroposterior Polarity

The anteroposterior polarity of the mouse embryo is not morphologically distinguishable until gastrulation at embryonic day 6.5 when the site of primitive streak formation unequivocally defines the posterior of the embryo. Recent work has revealed that well before the onset of gastrulation, the mouse embryo initiates dynamic and asymmetric patterns of gene expression in the visceral endoderm, an extraembryonic cell layer that comprises a squamous epithelium that surrounds the epiblast. Even though this extraembryonic tissue does not contribute to the body itself, it is involved in axis formation with the anterior visceral endoderm (AVE) being essential for anterior patterning of the embryo.

Work involving the labeling of individual cells of the blastocyst with EGFP, followed by in vitro culture and/or re-implantation and subsequent time-lapse imaging at gastrula stages has revealed that the spatial distribution of cells within the visceral endoderm can be traced back to polarity present at the blastocyst stage (for review, see Beddington and Robertson 1998). The distribution of visceral endoderm cells reflects characteristic cell movements. At pre-gastrula stages cells of the AVE are located at the distal tip of the embryo, but they then are believed to move unilaterally to the future anterior and providing the first overt break in the symmetry of the embryo. This migration has recently been investigated in transgenic mice where the regulatory elements of the Hex gene have been used to drive EGFP expression (Rodriguez et al. 2001; Srinivas et al. 2004). By culturing transgenic embryos on a microscope stage and acquiring time-lapse images it was possible to follow the migration of the AVE. Interestingly AVE cells were shown to continuously change shape and project filopodial processes in their direction of motion, suggesting that they are actively migrating. Additionally it was established that AVE cells migrate

as a single layer that is in continuous contact with the underlying epiblast, perhaps suggesting that this tissue might provide additional directional cues.

5.2 Primordial Germ Cell Migration

The primordial germ cells (PGCs) are the precursors of the gametes in mice. They arise from cells that also give rise to the proximal part of the allantois and are routinely visualized as an alkaline phosphatase positive cell population within the primitive streak and developing allantois. By embryonic day 8.5, they become embedded in the hindgut epithelium and thereafter migrate to the genital ridges and become incorporated into the developing gonads (for review, see Bendel-Stenzel et al. 1998).

In light of the uncertainty concerning the origin and migratory route taken by PGCs, Anderson, et. al. (2000) used regulatory elements for the Oct4 gene to drive EGFP in primordial germ cells during their genesis and subsequent migration. Time-lapse microscopy was used to selectively follow the trajectory taken by primordial germ cells. From this work they proposed a revised model of PGC formation and behavior, suggesting that only the cells in the ventral posterior primitive streak contribute to the germline and that the cells contained within the proximal allantois remain outside of the embryo proper and will not contribute.

The same group of investigators (Bendel-Stenzel et al. 2000) went on to use the Oct4::EGFP transgenic mice to image the later stages of PGC migration from the hindgut to the genital ridges, where they were observed to coalesce with each other and with somatic cells to form the primary sex cords. The change in migratory path and behavioral properties of these cells has been linked to the differential expression of several members of the cadherin class of adhesion molecules.

5.3 Imaging erythroblast differentiation and vascular development

The culture of 8.5 dpc embryos has been used to visualize the formation of the early cardiovascular system (Jones et al, unpubl.). At around 7.5 dpc, isolated clusters of blood, surrounded by endothelial cells form in the yolk sac of the embryo. These clusters expand and interconnect in order to form a random network, known as the capillary plexus. This plexus connects to the heart and as the heart begins to beat, the plexus is remodeled into a more mature, tree-like vascular system (reviewed in Flamme et al. 1997). Using a mouse that expresses GFP driven by the ε-globin promoter (Dyer et al. 2001), it is possible to image the formation of primitive erythroblasts and visualize changes in the vasculature. Endothelial cells can also be observed directly using a mouse that expresses GFP driven by the Tie2 promoter (Motoike 2000). Both markers are being used to image erythroblast differentiation, the formation of the capillary plexus, as well as the subsequent remodeling of the capillary plexus (Figure 1, panel 3). In addition to observing changes in morphology, we have also used the ε-globin::GFP mice to quantify flow velocities in the yolk sac as blood flow begins (Jones et al. submitted).

6 Advantages and limitations

A clear advantage to optical imaging in mouse embryos comes from the ability to resolve the cellular and sub-cellular events that occur during normal development or are a consequence of genetic alterations. Many mouse models are used to understand the etiology and progression of human diseases and birth defects and more tools are always welcome that provide greater mechanistic insight. Although other imaging modalities such as ultrasound, optical coherence tomography and MRI can provide insights into in utero development, these methods lack the cellular and even sub-cellular resolution offered by optical imaging. Also, the number of available fluorescent probes that can be used to identify specific cells or assay cell functions extends into

the thousands, while other methods suffer from the lack of available contrast agents. Thus, culture methods for mouse embryos brings together advanced genetic tools with imaging tools.

The culture of mammalian embryos is however more technically difficult than more traditional models for imaging development. Mammalian embryos are exceedingly sensitive to temperature fluctuations, media content and physical pressure. A constant temperature must be maintained at 38 °C in order for proper growth to occur. Quality rat serum is essential for growth and at 8.5 dpc, even slight pressure on the yolk sac causes circulation to the yolk sac to be terminated and the embryos will not turn and will not advance at a normal rate. The sensitivity of mammalian embryos to culture conditions often causes time-lapse experiments to fail, requiring several attempts at imaging to obtain a time-lapse sequence of a healthy embryo.

Movement during time-lapse imaging can be problematic in any organism. This can make it difficult to do quantitative measurements or track cells. Of the immobilization techniques described here, only the holding pipette offers significant control over the orientation of the embryo. Also, significant growth of the mammalian embryo between 7.5 and 9.5 dpc makes it difficult to keep all areas of interest in focus so the choice of the best objective lens for the resolution and magnification required can be important.

The cup-shaped configuration and turning of the mouse embryo also increases the complexity in imaging and culturing these embryos. The curvature of the embryo increases the number of optical sections needed to image a given region. As well, cell movements are more complex to understand since they tend to occur across different optical sections. As the embryo increases in size, the radius of curvature is also in constant flux. These problems are not isolated to mouse embryos, however, and can be overcome using 3D software for reconstructing and projecting the data and/or multi-photon imaging for improved depth penetration.

The strengths of mouse embryology, both as a human model and as a genetic model, make dynamic imaging of mouse development indispensable. Whole embryo culture of mammalian embryos for imaging combines molecular biology, genomics and biological imaging, offering a system for understanding the interplay of cell-cell communications and the signaling components important for orchestrating complex developmental events.

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9 Figure Legends

Figure 1 - GFP expression (green) driven by various promoters. Tbx6 expresses in the presomitic mesoderm and newly-formed somites. Oct4 expression can be seen in the primordial germ cells. ε-globin marks the primitive erythroblasts. Tie2 is a marker of endothelial cells. Alpha-fetoprotein (AFP) expression is seen in extra-embryonic tissues. Nkx2.5 expression is seen throughout the developing heart region.

Figure 2 - Fluorescent protein expression can be localized throughout the cytoplasm simply by expressing the protein. To localize proteins to the cell membrane, the fluorescent protein can be myristoylated. For localization to the nucleus, a fusion protein can be made of the fluorescent protein and histone 2 B.

Figure 3 - Preparation of rat serum, locating the dorsal aorta

Figure 4 - Heater box diagram

Figure 1

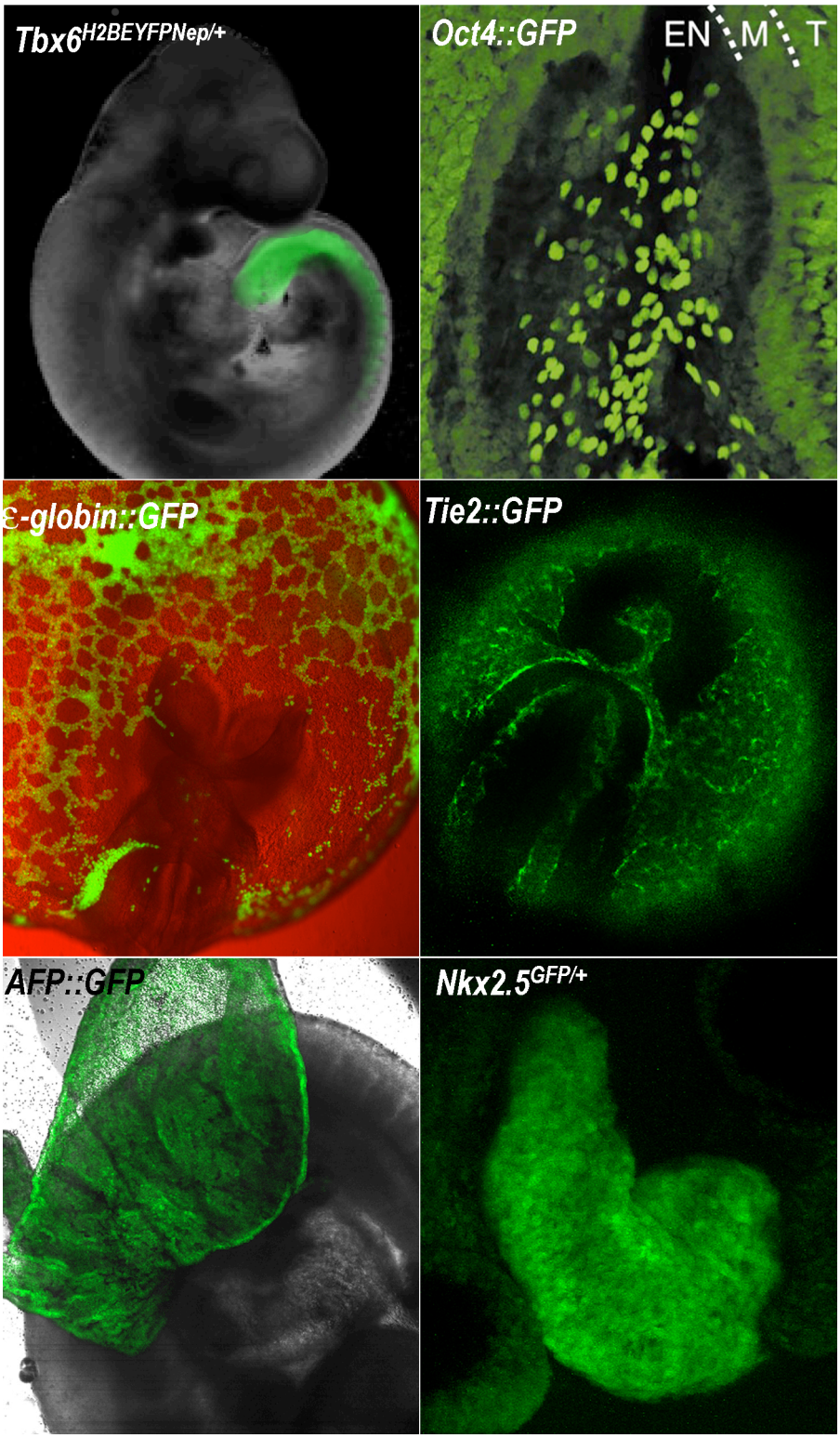
Tbx6H2BEYFPNep/+
Oct4::GFP
EN
M
T
ε-globin::GFP
Tie2::GFP
AFP::GFP
Nkx2.5GFP/+

Figure 2

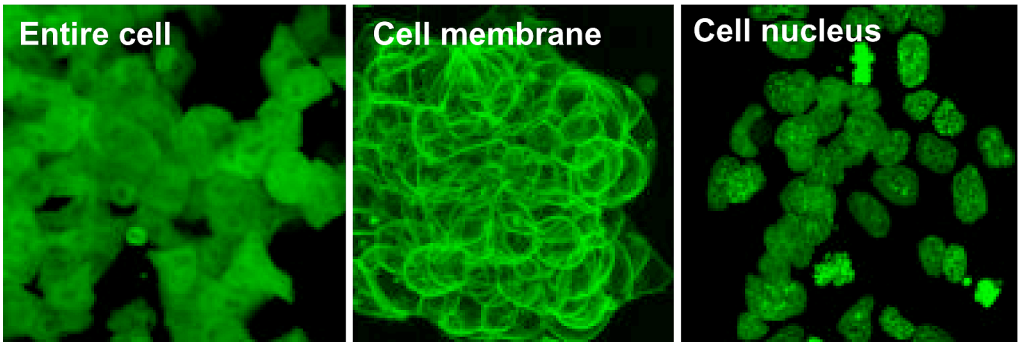

Figure 3

Use image from the Manipulating the Mouse Embryo, Figure 5.16a.

Figure 4

Use image from the Manipulating the Mouse Embryo, Figure 5.17.